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TVX 2706 – a new phosphodiesterase inhibitor with anti-inflammatory action
Biochemical characterization

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Abstract

The effects of the anti-inflammatory and analgesic drug 3-ethyl-1-(3-nitrophenyl)-2,4[1H, 3H]-quinazolindione (TVX 2706) on neuronal and glial cell culture systems including neuroblastoma × glioma hybrid cells have been studied. This compound strongly enhances the increase in intracellular levels of cyclic AMP caused by appropriate effectors in all systems tested so far. EC_{50} values are in the submicromolar range. The effect is apparently neither due to an increased responsiveness of the hybrid cells for an effector like prostaglandin E_1 nor to an increased activity of adenylate cyclase, but to an inhibition of both low and high affinity cyclic AMP phosphodiesterases. Half-maximal inhibition of enzyme activity is obtained at 10 μM TVX 2706. The drug is at least equipotent to or more potent than some other common phosphodiesterase inhibitors. Inhibition of phosphodiesterase activity is also observed in homogenates from rat polymorphonuclear leucocytes, where the low K_m-enzyme is preferentially inhibited. TVX 2706 does not interfere with the calmodulin activation of phosphodiesterase. The role of phosphodiesterase inhibition as a possible mechanism of the anti-inflammatory action of TVX 2706 is discussed.

Introduction

Inflammatory processes have been suggested to be mediated by a variety of substances arising from arachidonic acid metabolism, e.g. prostaglandins and leucotrienes. Inhibition of the synthesis of prostaglandins is generally accepted as a mechanism of action of nonsteroidal anti-inflammatory drugs [1]. However, there is now substantial evidence that cyclic nucleotides (cyclic AMP or cyclic GMP) might also play an important role in inflammation (reviewed in [2]) and immune responses. The exact nature of these processes has not yet been elucidated, but it may be suggested that drugs which alter the levels of cyclic nucleotides might also exert an influence on these systems.

TVX 2706 (3-ethyl-1-(3-nitrophenyl)-2,4[1H,3H]-quinazolindione) has been shown to be a very potent analgesic and anti-inflammatory drug in several animal models of inflammation [3]. The structure of TVX 2706 is shown in Fig. 1.

Moreover, this drug efficiently inhibits the aggregation and chemotaxis of polymorphonuclear leucocytes *in vitro* and the release of lysosomal enzymes [4]. These three processes are inhibited by elevated levels of cyclic AMP [5–7].

Therefore, it is tempting to speculate that TVX 2706 might influence the intracellular levels of cyclic AMP. In the present study we have investigated the effects of TVX 2706 on cyclic AMP levels in neuronal and glial cell culture systems and on the enzymes involved in cyclic AMP metabolism i.e. adenylate cyclase and phosphodiesterase (PDE). Tissue culture cells offer the possibility to study drug effects in living cells under well defined conditions. In this context the use of cell cultures in investigating receptor mediated responses was of special interest.

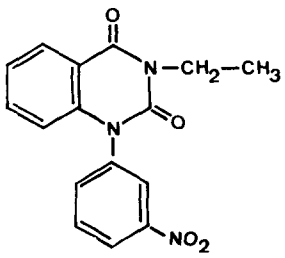

Figure 1
Formula of TVX 2706.

Neural cultures – clonal cell lines or primary brain cultures – have advantageously been applied for studying the control of intracellular cyclic nucleotide concentration at the receptor or enzyme level [8].

Materials and methods

The rat glioma cells C6-BU-1, the bromodesoxyuridine-resistant mutant of wild type C6-cells [9] and the neuroblastoma × glioma hybrid cells 108CC15 [8] were cultured as described previously [9]. The method for obtaining primary cultures from embryonic rat brain by mechanical dissociation was identical to that reported by VAN CALKER et al. [10]. Rat cortical slices were prepared and incubated according to TÜRK [11].

For determining effects of hormones and drugs on cyclic AMP levels incubation of cells and the subsequent assay of cyclic AMP were performed as detailed elsewhere [12, 13]. The activity of adenylate cyclase in cell homogenates using α^{32}P-adenosine triphosphate as substrate was measured according to SABOL and NIRENBERG [14].

3′,5′-Cyclic AMP phosphodiesterase activity in homogenates from various sources was determined by the method of THOMPSON and APPLEMAN [15] with modifications as indicated. Cells were prewashed in Puck's medium D1 supplemented with glucose and sucrose before homogenization. Tissue was homogenized directly in the homogenization buffer containing 40 m*M* Tris-HCl, 10 m*M* $MgCl_2$, pH 8.0 (37°C). The incubation mixture (total volume 200 μl) contained 40 m*M* Tris-HCl, pH 8.0 (37°C), 50 m*M* $MgCl_2$, about 44,000 d.p.m. ^{3}H-cyclic AMP, unlabelled cyclic AMP (in some experiments ^{3}H-cyclic GMP and unlabelled cyclic GMP) and 50 to 100 μg protein. In order to study both the high and the low affinity PDE two concentrations of cyclic AMP were used: 3 μM (high) and 100 μM cyclic AMP (low). After incubation at 37°C for 45 min the reaction was stopped by freezing the samples in ethanol-dry ice. The phosphodiesterase was inactivated by boiling for 5 min, 0.2 U (20 μl) 5′-nucleotidase from snake venom (*Crotalus atrox*) was added to each tube and incubated for 10 min at 37°C. The reaction was stopped by addition of 1 ml ice-cold 4 m*M* acetic acid. After centrifugation 610 μl from each tube were applied to AG1-X2 anion exchange columns (glass pasteur pipettes) which were eluted with 4 ml 4 m*M* acetic acid. Eluates were collected in scintillation vials and radioactivity determined at 30% counting efficiency. Recovery of reaction product was measured with ^{14}C-adenosine and was >95%. Enzyme activity was linear with time up to 60 min and with protein up to 200 μg.

For determination of calmodulin activation of PDE the experimental protocol was modified. The reaction mixture containing 0.1 *M* glycylglycine buffer pH 7.5 (25°C), 3 m*M* $CaCl_2$, 1.2 m*M* $MgCl_2$, 1 mU PDE, and calmodulin (dissolved in 9 m*M* 2-*N*-morpholino-ethane sulfonic acid buffer (MES), 0.1 m*M* $CaCl_2$, 100 m*M* NaCl, pH 6.5, and diluted with MES/bovine serum albumin (1 mg/ml)) was preincubated at 30°C for 10 min. The incubations were commenced by adding 86.4 μM cyclic AMP and continued for 20 min at 30°C. The subsequent steps were as described above. Usually determinations were made in duplicates with variations <10%. Experiments were reproduced at least twice.

Protein was measured according to the method of BRADFORD [16]. TVX 2706 was dissolved in dimethylsulfoxide (DMSO). The maximal possible concentration in aqueous medium is 70 μM.

Materials used in this study were from the following sources: ^{14}C-adenosine (58 mCi/mmol) and ^{3}H-cyclic AMP (34.5 Ci/mmol), New England Nuclear, Dreieich; calmodulin, Boehringer, Mannheim; isobutylmethylxanthine, Serva, Heidelberg; isoproterenol, 5′-nucleotidase from snake venom, grade IV, and papaverine, Sigma, Munich; phosphodiesterase (activator-deficient from bovine heart), Boehringer, Mannheim; prostaglandin E_1, Upjohn, Kalamazoo; RO 20-1724, Hoffmann-La Roche, Grenzach; rolipram (ZK 62711), Schering, Berlin; trifluoperazine, Boehringer, Mannheim.

Table 1
Influence of TVX 2706 on intracellular cyclic AMP levels of cell culture systems and rat cortical slices in the absence and presence of various hormones.

Additions (μM)	Type of cells			Cortical slices[d]
	C6-BU-1 glioma	108CC15 hybrid	Primary culture	
		cyclic AMP (pmol · mg^{-1} protein)		
DMSO 0.5%	13 ± 8	8 ± 2	9 ± 3	12 ± 0.6
TVX 2706 (10)	23 ± 12	24 ± 8	9 ± 5	29 ± 14
Isoproterenol (1)	1505 ± 235		2549 ± 371[b]	32 ± 3[c]
Isoproterenol + TVX 2706	3473 ± 319		3989 ± 594[b]	63 ± 13
Adenosine (100)		67 ± 7[a]	135 ± 23	
Adenosine + TVX 2706		1587 ± 60[a]	678 ± 56	
PGE_1 (1)		706 ± 34	354 ± 99	
PGE_1 + TVX 2706		3268 ± 231	771 ± 9	

[a] Adenosine concentration 10 μM.
[b] Isoproterenol concentration 0.1 μM.
[c] Isoproterenol concentration 100 μM.
[d] Values are the mean of slices from 3 animals ± SEM.
Cyclic AMP values are the mean of triplicates ± SEM.

Results

In the two cell lines tested, the C6-BU-1 glioma cells and the 108CC15 neuroblastoma × glioma hybrid cells as well as in primary cultures from embryonic rat brain, cyclic AMP levels are raised by several effectors (Table 1). TVX 2706 (10 μM) strongly enhances this cellular response. The action of adenosine on hybrid cells is particularly amplified (25-fold, Table 1). Qualitatively similar results have been obtained with rat cortical slices as with C6-BU-1 cells. By plotting the augmentation of both prostaglandin E_1 (PGE_1) and adenosine activity on the cyclic AMP levels in hybrid cells as a function of TVX 2706 concentration, an EC_{50}-value of about 0.4 μM can be determined graphically (see Fig. 2).

We performed a variety of experiments to examine the molecular mechanism of this TVX 2706 action. In the presence of the drug the sensitivity of the receptor-cyclase complex e.g. for PGE_1 is apparently not changed. In the absence and presence of TVX 2706 the EC_{50} values are 0.3 and 0.2 μM, respectively (Fig. 3).

The cyclic AMP synthesizing enzyme adenylate cyclase was measured under several conditions. TVX 2706 fails to stimulate cyclase activity over control values either in the absence or in the presence of PGE_1, adenosine or sodium fluoride (Table 2).

Another possible mechanism by which cyclic AMP can be elevated is via inhibition of

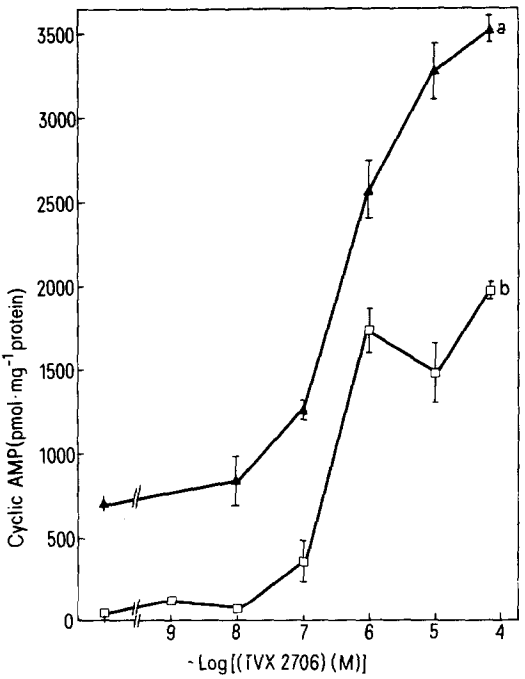

Figure 2
Concentration dependent increase by TVX 2706 of cyclic AMP production in hybrid cells in the presence of hormones. Neuroblastoma × glioma cells were incubated in the presence of 1 μM PGE_1 (curve a) or 10 μM adenosine (curve b) with the concentrations of TVX 2706 indicated before the intracellular cyclic AMP concentration was determined. Each point represents the mean of triplicates ± SEM.

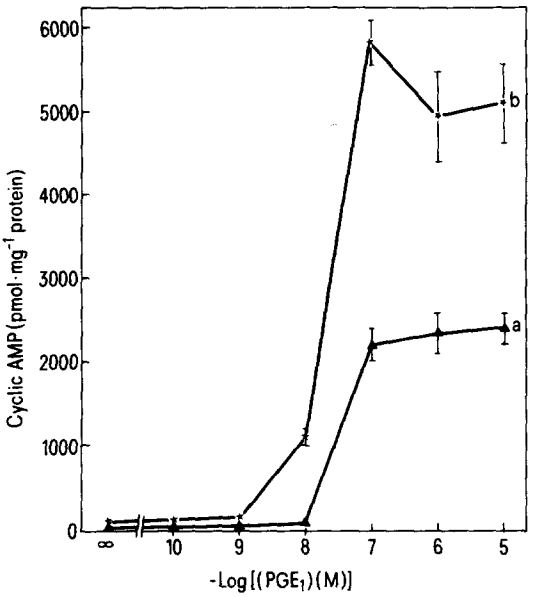

Figure 3
Influence of TVX 2706 on the elevation of cyclic AMP concentration caused by PGE_1. Hybrid cells were incubated in the presence of 0.5% DMSO (curve a) or 10 μM TVX 2706 (curve b) with various concentrations of PGE_1. Each point represents the mean of triplicates ± SEM.

Table 2
Influence of TVX 2706 on adenylate cyclase activity in hybrid cells.

Additions (μM)	^{32}P-cyclic AMP formed (pmol · mg^{-1} protein · min^{-1})
0.5% DMSO	19.1 ± 1.8
TVX 2706 (10)	17.3 ± 0.3
Adenosine (100)	42.3 ± 1.1
Adenosine + TVX 2706	46.1 ± 0.1
Sodium fluoride (10,000)	42.8 ± 8.4
Sodium fluoride + TVX 2706	45.8 ± 0.1
PGE_1 (10)	173.3 ± 3.9
PGE_1 + TVX 2706	162.9 ± 14.4

Values are the mean of duplicates ± SEM.

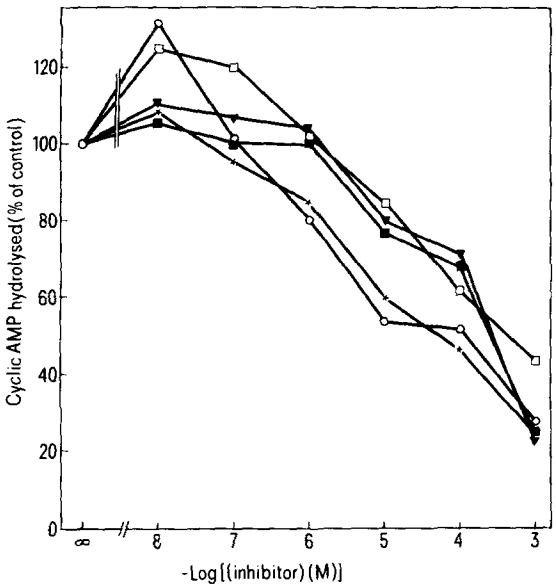

Figure 4
Effects of various PDE inhibitors on PDE activity in hybrid cell homogenates. PDE was measured as described in 'Materials and methods'. Inhibitors were dissolved in DMSO (final concentration 0.1% or 10%). Cyclic AMP concentration was 100 μM. *——* TVX 2706; O——O rolipram; ■——■ RO 20-1724; ▼——▼ isobutylmethylxanthine; □——□ papaverine.

cyclic AMP PDE. In fact, TVX 2706 markedly inhibits PDE activity in hybrid cell homogenates (Fig. 4). In comparison to other widely used PDE inhibitors, TVX 2706 is equipotent with rolipram and approximately 10 times more potent than RO 20-1724, isobutylmethylxanthine and papaverine (Fig. 4). The IC_{50} value of TVX 2706 is 10 μM.

Kinetic analysis of the PDE activity reveals at least two forms of PDE present in the cells with K_m-values of 23 and 100 μM (Fig. 5, curve a). In the presence of TVX 2706 these K_m-values are unchanged, thus indicating a noncompetitive type of inhibition (Fig. 5, curve b). Moreover, under this condition a third high affinity PDE with a K_m-value of 3 μM can be detected. Linearity in the Lineweaver-Burk-plot is achieved down to 0.5 μM. When measured at 3 μM cyclic AMP the high affinity PDE is inhibited by TVX 2706 to the same extent as at 100 μM indicating that both the high and low affinity PDE are affected (not shown).

Since TVX 2706 is an anti-inflammatory drug and since polymorphonuclear leucocytes (PMN) play a crucial role in inflammatory processes, we studied the action of the drug on PMN PDE. In contrast to the effect in hybrid cell homogenates, TVX 2706 has only a small effect on the low affinity PDE (Fig. 6 A), but markedly inhibits the high affinity enzyme (Fig. 6 B). The dose-dependent curve (Fig. 6 C) is comparable to that seen with hybrid cell homogenates (Fig. 4). Qualitatively similar results are obtained with homogenates from calf cortex and calf heart (data not shown).

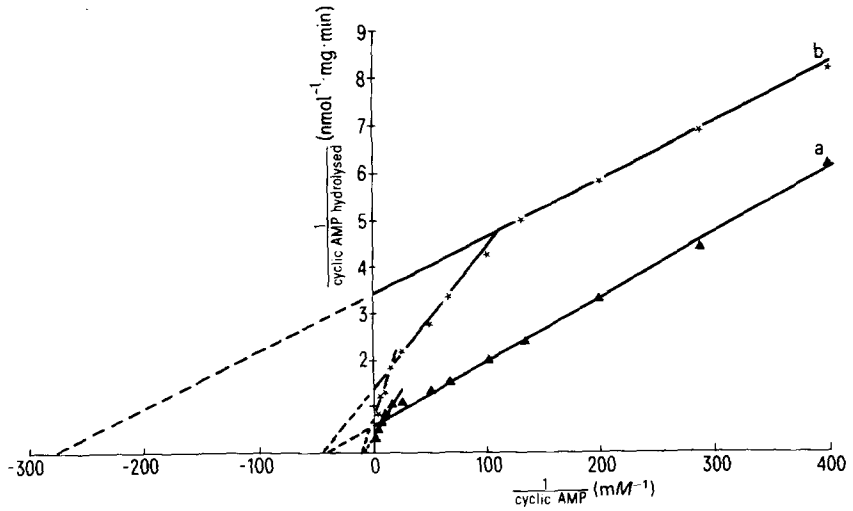

Figure 5
Lineweaver-Burk plot of substrate-dependency of cyclic AMP hydrolysis in hybrid cell homogenate. a: in the presence of 0.1% DMSO; b: in the presence of 10 μM TVX 2706.

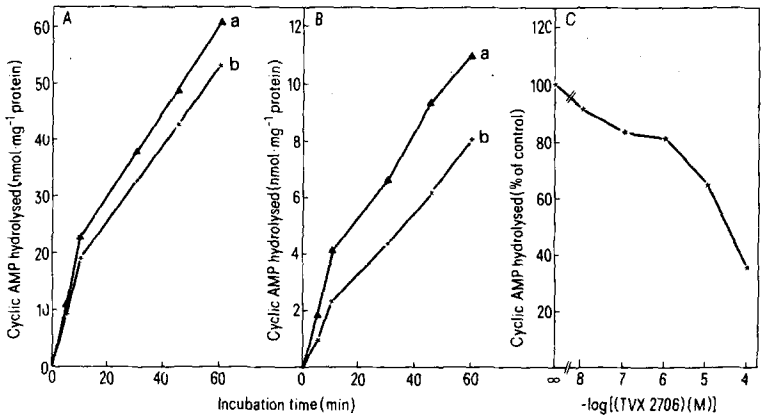

Figure 6
Influence of TVX 2706 on PDE activity in rat PMN. Rate of cyclic AMP hydrolysis was measured in the presence of 0.1% DMSO (curve a) or 10 μM TVX 2706 (curve b) at 100 μM (panel A) or 3 μM (panel B) cyclic AMP. Concentration dependent inhibition of PDE activity by TVX 2706 at 3 μM cyclic AMP (panel C).

Calmodulin is known as an activator of some phosphodiesterases [17]. Although drugs shown to be calmodulin inhibitors like trifluoperazine, chlorpromazine and haloperidol [18, 19] fail to inhibit hybrid cell PDE, we investigated the effect of TVX 2706 on the activation of commercially available calmodulin-sensitive PDE by calmodulin. The activator stimulates PDE activity nearly 5-fold already at very low concentrations (Fig. 7, curve a). 10 μM TVX 2706 cannot prevent this activation (curve b), whereas 10 μM trifluoperazine almost completely blocks it (curve c). The slight effect of TVX 2706 appears to be caused by a small inhibition of enzyme activity as it is seen also in the absence of calmodulin. These results indicate that TVX 2706 does not affect the calmodulin-sensitive PDE.

We also examined the influence of TVX 2706 on cyclic GMP hydrolysing activity in hybrid cell homogenates at both low (3 μM) and high (170 μM) cyclic GMP concentrations. Only a very small (20%) if any inhibition at 10 μM TVX 2706 on cyclic GMP PDE was observed (data not shown). Therefore, TVX 2706 appears to be a highly selective inhibitor of cyclic AMP PDE.

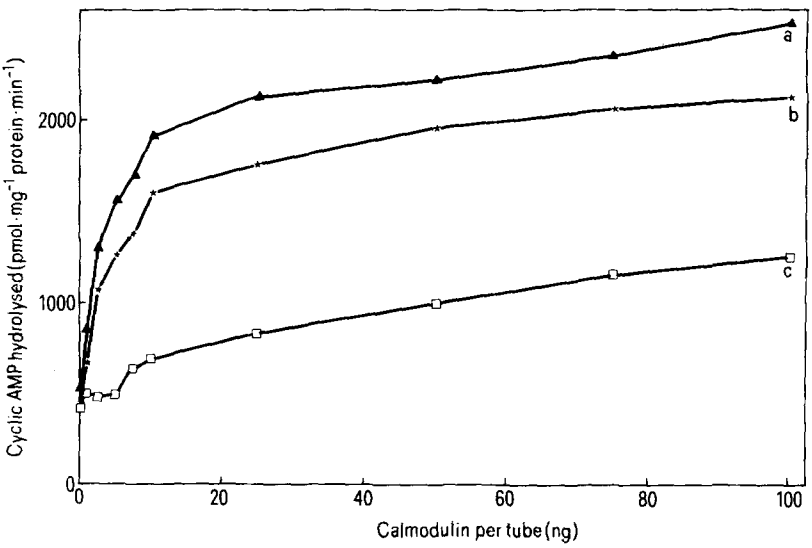

Figure 7
Effect of TVX 2706 and trifluoperazine on activation by calmodulin of activator-free PDE from bovine heart. PDE-activity (1 mU per tube) was determined in the presence of 0.1% DMSO (curve a) or 10 μM TVX 2706 (curve b) or 10 μM trifluoperazine (curve c). Cyclic AMP concentration was 86.4 μM.

Discussion

In the present study we have shown that the very efficient anti-inflammatory drug TVX 2706 strongly enhances cyclic AMP responses in all cellular systems tested so far. Several possibilities exist to explain this observation:

(1) TVX 2706 acts via own adenylate cyclase coupled receptors or via a direct stimulation of the enzyme. The latter mechanism can be excluded by the lack of an effect of TVX 2706 on cyclase activity. This missing effect on adenylate cyclase clearly differentiates TVX 2706 from substances like forskolin. Like TVX 2706, this diterpene augments adenylate cyclase coupled receptor-mediated responses [20]. However, the augmentation by forskolin is due to a stimulatory effect on the catalytic subunit of adenylate cyclase while having little effect on PDE [20].
(2) TVX 2706 acts via a modulation (e.g. sensitization) of hormone receptors. This is not the case, because EC_{50}-values (Fig. 3) are not changed by TVX 2706.
(3) TVX 2706 acts by inhibiting cyclic AMP degradation by PDE.

Our results clearly show that TVX 2706 is indeed a selective cyclic AMP PDE inhibitor in homogenates from various sources including PMN cells. In potency it is comparable to rolipram but more potent than the other reference drugs tested.

The EC_{50} value of TVX 2706 and other PDE inhibitors on cyclic AMP levels in intact cells is more than 20-fold lower than the IC_{50} value for PDE inhibition measured in the homogenates. This difference might be caused by a high sensitivity of PDE to homogenization and/or freezing of the homogenate. Similarly a loss of sensitivity to RO 20-1724 of erythrocyte PDE after freezing has been reported [21].

Of the various effectors tested in the presence of TVX 2706, the action of adenosine is most amplified both in the neuroblastoma × glioma hybrid cells and in the primary cultures. This may have some implication for the anti-inflammatory action of TVX 2706 as will be mentioned below. The reason for the preferential effect on adenosine action is not yet clear.

The drug appears as a noncompetitive inhibitor of low and high affinity PDEs depending on the systems used. With the drug present, a high affinity PDE seems to be resolved in the Lineweaver-Burk plot not seen in the absence of TVX 2706. One way to explain this result is, that the activities of the enzymes with higher K_m-values (23 and 100 μM) are negligible at low substrate concentrations in the presence of TVX 2706. Under these circumstances the high affinity enzyme might contribute most to the total activity. Alternatively, TVX 2706 might inhibit the intermediate K_m-enzyme (23 μM) in an uncompetitive manner resulting in a parallel shift of the corresponding line in the Lineweaver-Burk plot. If one assumes that at very low substrate concentrations (beyond those indicated in Fig. 5, that is down to 0.5 μM) the activity of the low and intermediate affinity PDE is negligible, then one should observe the high affinity PDE in the Lineweaver-Burk plot at low cyclic AMP concentrations. However, this is not the case and supports this second explanation. Transformation of the data according to Eadie-Hofstee reveals a similar result. Only purification of the different enzymes can help to clarify the problem of how many PDEs are present and which of them are inhibited by TVX 2706.

TVX 2706 expresses marked selectivity for the cyclic AMP PDE. Such strong selectivity has also been observed with several other PDE inhibitors e.g. with RO 20-1724 and rolipram, whereas papaverine shows only weak selectivity [22].

At least three forms of PDEs were reported to exist in most mammalian tissues which differ in substrate specificity and kinetic parameters (for review see [17]). The PDE with low affinity for cyclic AMP but high affinity for cyclic GMP appears to be dependent on calmodulin [17]. A calmodulin-independent PDE hydrolyses cyclic AMP as well as cyclic GMP [17]. Our experiments clearly show that TVX 2706 does not act as calmodulin-inhibitor or cyclic GMP PDE inhibitor:

(1) In the cell homogenates where TVX 2706 inhibits PDE activity, the calmodulin antagonists trifluoperazine, chlorpromazine and haloperidol [18, 19] are practically inactive, thus indicating that under our conditions mainly calmodulin-insensitive PDE activity is measured. The cloned glioma C6 and the neuroblastoma N18 cell lines, the wild-type forms of the parental cell lines of the hybrids 108CC15, have been found to contain only low calmodulin activable PDE activity [23].

(2) Unlike trifluoperazine, TVX 2706 does not prevent activation by calmodulin of purified PDE (Fig. 7).
(3) If TVX 2706 were a calmodulin-antagonist, then it should influence cyclic GMP hydrolysis [17]. This is hardly the case either at low or high substrate concentrations.
tions.

The data also demonstrate an effect of the drug preferentially on the high affinity PDE of PMN suggesting elevated cyclic AMP concentrations. The increased cyclic AMP level in turn might influence several cellular processes which are important for the onset of inflammation such as chemotaxis, aggregation and lysosomal enzyme release and which are influenced by TVX 2706 as detailed in Ref. [4].

In addition, an increase in intracellular cyclic AMP turns off the release of mediators of inflammation as histamine, leucotrienes and others, whereas a decrease stimulates the release [24]. Adenosine elevates leucocyte cyclic AMP and therefore inhibits mediator release [24]. Consequently the enormous enhancement by TVX 2706 of adenosine action might have a dramatic inhibitory effect on the release of mediator substances. Moreover, according to the hypothesis put forward by Lapetina [25], cyclic AMP regulates the phosphatidylinositol cycle leading to lowered levels of free arachidonic acid, the metabolites of which play an important role in the inflammation.

Taken all together, an elevated cyclic AMP content in inflammatory cells might cause anti-inflammation by inhibiting several reactions, which are independent of each other. This fact could explain the excellent potency of TVX 2706 *in vivo* as an anti-inflammatory drug. That inhibition of PDE activity in fact causes anti-inflammation is supported by experiments showing that other PDE inhibitors are also anti-inflammatory active in *in vivo* model systems (Pelster et al., personal communication).

Furthermore SILVOLA et al. [26] reported that a variety of nonsteroidal anti-inflammatory drugs act as inhibitors of both cyclic AMP and cyclic GMP PDE. Most of these drugs have been shown to have gastric side effects [27]. However, it has been proposed, although not yet clearly proven, that drugs preferentially inhibiting cyclic AMP PDE and not cyclic GMP PDE would have fewer side effects [28]. The demonstrated selectivity of TVX 2706 for cyclic AMP PDE might therefore be an advantage of this drug.

The inverse relationship between cyclic AMP content and effector function in immune and inflammatory cells has mainly been observed using animal cells. It has been reported that human PMN unlike human lymphocytes respond only marginally to agonists which are generally thought to elevate cyclic AMP [29]. On the other hand, stimulation of human PMN with the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine leads to a short-lasting increase in cyclic AMP [30] which might in the presence of TVX 2706 be sufficient to inhibit the cellular processes mentioned above. Further studies are necessary to prove the inhibition by TVX 2706 of PDE activity as the pharmacological mechanism of action. We cannot yet exclude that this drug also affects other mechanisms important in inflammation as well.

PDE inhibitors as a therapeutical tool are currently under investigation in several laboratories and clinics. For example, RO 20-1724 (and also rolipram) is being developed for its antipsoriatic activity [31], and rolipram has been reported to reverse experimental produced amnesia and to facilitate memory [32]. Since TVX 2706 is very similar to these two PDE inhibitors in terms of cyclic nucleotide selectivity and potency, one could envisage TVX 2706 as a therapeutic drug for other indications besides inflammation.

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